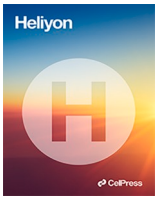
Heliyon 10 (2024) e24046

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Structural dependence of concentrated skim milk curd on micellar restructuring

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ARTICLE INFO

Keywords:
Concentrated skim milk
Renneting
Slow acidification
Rheological properties
Diffusion coefficient
Micellar restructuring

ABSTRACT

This study was conducted to establish an understanding of how milk concentration modulates the rennet curd structure. Rennet-induced gelation and renneting under slow acidification achieved using glucono-δ-lactone (GDL) and structural properties of reconstituted skim milk gels at two concentration levels (9 and 25 % total solids) were studied by measuring variations in (a) viscoelastic behaviour, (b) micellar size, charge density, diffusivity, and (c) hydrophobicity using dynamic rheometry, dynamic light scattering and fluorimetry, respectively. Concentrated milk showed a greater estimated hydrodynamic radius of casein micelles, lower zeta (ζ)-potential, ratio of serum to total Calcium (Ca) and charge density and increased surface hydrophobicity, all supporting the view that micellar restructuring particularly sub-particle transfer takes place and contributes to rapid gelation. Moreover, hydrophobic interactions occurred very quickly (within 5 min in combined gels, 10 min for renneting only), demonstrating their pivotal role during the flocculation stage. All gels exhibited a solid viscoelastic character as the elastic modulus (G') was greater than loss modulus (G'') while both G' and tan δ (G''/G') were frequency-dependent. Frequency sweeps classified the concentrated gels into three stiffness categories caused by the level of rennet or GDL as rigid, hard and soft, whereas an increased flow-like behaviour (high tan δ), restricted diffusion and excessive water retention revealed limited structural rearrangements (contraction & macrosyneresis) during curd ageing. Acidification increased the diffusion rate in control curd, thus, enhanced contractive rearrangements, macrosyneresis and curd strength. Findings suggest that micellar restructuring induced by milk concentration is the principal modulator of the curd structure.

1. Introduction

In order to improve cheese quality or overcome inconsistencies due to natural variations in milk composition, intentional modifications are made [1]. The type of milk system selected as the starting material and the concentration level have different effects on curd structure, and consequently determine the qualities of both cheese and whey products. Guinee, Pudja & Mulholland [2] and Ong et al. [3] found excessive losses of fat and protein into whey when milk was concentrated to protein content over 5 %, and this was attributed to a porous gel structure. In addition to milk composition, cheese making conditions such as temperature, renneting extent and pH can also modify the curd structure [4–8].

Several studies have attempted to explain why milk composition and processing conditions affect gel structure. These include, for

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https://doi.org/10.1016/j.heliyon.2024.e24046
Received 1 April 2023; Received in revised form 23 November 2023; Accepted 2 January 2024
Available online 3 January 2024

example; the dependence of curd strength on native casein micelle size [9], demineralisation of Ca and a lower net charge reported as the reason for a reduced density of the protein network during renneting of milk with decreasing pH [10] and a higher gelation temperature reported to be responsible for an unstructured and coarse network of larger casein aggregates [11]. Rearrangements in rennet gels from pre-heated milk were found to be limited due to crosslinking by denatured whey proteins [6], while using a fractal scaling model, Mellema [12] reported that the structure of skim milk curd at various pH and temperatures is related to rearrangements thought to be caused by particle fusion.

Furthermore, the cheesemaking properties of milk systems concentrated by various methods have been extensively researched and structural or compositional differences reported [13–21]. However, studies on structural changes occurring within the gel as a function of milk concentration during combined renneting and acidification, so far, seem to be limited. The first report appeared a decade ago and was on quark-type cheese [20]. Similar studies on phosphocaseinate and acidified milk are also available [22–24] but there appears to be none on conditions applied for the manufacturing of hard cheeses. Therefore, the underlying reason why milk concentration modulates curd formation and structural properties appears to have not been clearly elucidated. Based on some evidence already available in literature that micellar restructuring (changes involving particle transfer, secondary structure, aggregate formation & size increment) occurs during milk concentration [18,25–30], the present research attempted to establish its relationship to structural properties of renneted skim milk gel with varying concentration levels of total solids (9 and 25 %), examined by dynamic rheometry and dynamic light scattering measurements. Gelation was achieved by renneting or renneting combined with slow acidification (using GDL). In addition, changes in hydrophobic interactions and retention of different Ca fractions, ash, total solids and moisture into the curds were examined to further explain some structural rearrangements related to phase separation or syneresis [31].

2. Materials and methods

2.1. Preparation of reconstituted skim milk samples

Reconstituted skim milk samples were prepared from low heat skim milk powder (Warrnambool Cheese and Butter - Saputo, Warrnambool, Victoria, Australia) dispersed in Milli-Q water, stirred at room temperature (~20 °C) for at least 2 h and kept refrigerated overnight to fully hydrate the proteins. The dispersions were stirred and standardised to two concentration levels i.e., a concentrated skim milk of 25 % total solids (9.4 % protein) and a control of 9 % total solids (3.2 % protein). The concentrations were chosen as part of an undergoing project that covers the range of protein levels studied previously for the manufacture of Cheddar cheese [2]. Composition of skim milk during standardisation was tested using an ultrasonic milk analyser (Milkotronic Ltd, Nova Zagora, 8900 Bulgaria).

2.2. Preparation of rennet whey samples

Skim milk dispersions were coagulated in 50 mL tubes either by renneting only using a commercial calf rennet (290 International Milk Clotting Units (IMCU) mL^{-1}, Cheeselinks, Lara, Victoria, Australia) or renneting combined with slow acidification achieved by addition of glucono-δ-lactone (GDL). Milk samples were placed in a water bath and warmed to a coagulation temperature of 31 °C [32]. For combined renneting and acidification, GDL was added based on protein content (0.2 g/g) to mimic the slow acidification process of a starter culture, targeting a drainage pH of 6.0 ± 0.1, achieved after 1 h. Concentrated milk was divided into two sub-samples and rennet added to the first one based on milk volume i.e., the same as added to control (0.02 IMCU/mL) and to the second one based on protein content (0.47 IMCU/g). Samples were left undisturbed to coagulate for 30 min followed by cutting and then cooking at 38 °C for 30 min. Curd pressing was simulated by centrifugation for 1 h at 20 °C and 1700×*g* according to Shakeel-Ur-Rehman, McSweeney & Fox [33]. Another set of the same skim milk samples was also coagulated under the same conditions without GDL.

2.3. Total solids, ash and Ca fractions in milk and whey

Total solids, ash and three Ca fractions (total, serum and ionic Ca) were assessed on both milk and the resultant whey samples. Ten grams of each skim milk or whey were ultra-centrifuged for 1 h at 25 °C and 100000×*g* to obtain the serum fraction which was separately dried with another 10 g of the original milk or whey and ~2 g of pressed curd samples. Drying to a constant weight was performed in a hot airdrying oven at 105 °C to determine the total solids, and ash content determined by mineralising dry samples in a muffle furnace at 550 °C for at least 18h. The whole ash obtained was dissolved into 100 mL of 5 % nitric acid, filtered through 0.45 μm membrane filters and directly analysed using an inductively coupled plasma atomic emission spectrometer (Multitype, Shimadzu Corporation, Kyoto, Japan). Ionic Ca of skim milk and whey samples was measured using a Ca ion selective electrode of the laboratory research grade benchtop pH/mV/ISE meter – HI5222 series (Hanna Instruments Inc. Woonsocket, RI, USA). After addition of 500 μL ionic strength adjuster solution (HI 4004-00, Hanna Instruments Inc. Woonsocket, RI, USA) to 25 mL of each sample, records were taken with continuous stirring once stable values were displayed.

2.4. Dynamic rheometry measurements

The time course of gel formation was assessed by dynamic rheometry with a bob (25 mm diameter) and cup (27.11 mm diameter) geometry using a Physica MCR 301 rheometer (Anton Paar GmbH, Graz, Austria) as described previously [34] with some modifications. Each sample (19 mL) was loaded at 31 °C into a rheometer cup immediately after predetermined amounts of the coagulants

(rennet only or GDL + rennet) were added. Variations in the elastic (storage) modulus (G′) and the ratio of viscous (loss) to elastic moduli (G''/G′, also known as loss tangent or tan δ) with gelation time were monitored at constant temperature, frequency and strain of 31 °C, 1 Hz and 0.5 %, respectively. Rennet coagulation time (RCT) defined as the time at which G' ≥ 1 Pa [35], time to reach the cutting window defined according to Panthi et al. [19] as the time at which G' ≥ 35 Pa (k_{35}) and G′ after 30 min (G'_{30}, Pa) were all determined. After 1 h of gelation, curd properties were tested by frequency sweep (0.1–100 Hz) at a constant strain of 0.5 % which was within the linear viscoelastic region. Changes in G′, tan δ, complex viscosity (η*, Pa.s) and shear stress (τ, Pa) as a function of the oscillatory frequency were evaluated.

2.5. Dynamic light scattering and ζ-potential measurements

Dynamic light scattering measurements were performed on reconstituted skim milk samples prior to and during gelation. Both diffusion coefficients and ζ-potential were measured using a Nano-ZS Zetasizer (Malvern Instruments, Malvern, UK) as described previously [27] except that samples were not diluted. Previously, zeta potential was linearly correlated to the particle size [36] thus a ratio of effective zeta potential to estimated average hydrodynamic radius of skim milk particles is nominally termed charge density. Charge density in milk samples was calculated after estimating the average hydrodynamic size of micelles using Stokes-Einstein equation [37–39] shown below:

$$r_h = \frac{k_b T}{6\pi\eta \mathrm{D}} \quad \text{or} \quad d_h = \frac{k_b T}{3\pi\eta \mathrm{D}}$$

where r_h is the hydrodynamic radius and d_h is diameter, k_b is the Boltzmann constant, T is the temperature (K), η is the viscosity of the solvent and D is the measured diffusion coefficient.

This calculation was based on previous reports that dynamic light scattering instruments in backscattering mode at 173° or 180° can measure particle size in concentrated suspensions [40] up to 40 % [41]. In addition, de Kruif [38] and de Kruif & Zhulina [42] applied the same technique and presented changes in micellar size during renneting. Further, it has been noted that casein micelles in concentrated milk systems behave like colloidal hard spheres up to >45 % [43], with constant dynamic mobility up to 4 × concentration [44] and free diffusing Brownian motion up to a volume fraction of 0.3 [45]. The viscosity (at 10/s) of the serum phase (prepared by ultracentrifugation at 100000×*g* at 25 °C for 1 h) was used as its components (water, whey proteins and salts) are considered a continuous medium in which casein micelles viewed as hard spherical particles are dispersed [38] and since milk (≤30 % total solids) or whey protein solutions (≤10 %) exhibit Newtonian behaviour [46–48] a shear rate of 10/s would have no impact on viscosity. Liu et al. [49] used the viscosity of milk serum prepared at 55000×*g*, 25 °C for 90 min. Changes in diffusion coefficients during curd formation were also tested for 1 h and the time to reach a polydispersity index of 1 (PDI_1) was also recorded. PDI is an important physical property used to describe micellar size distribution and an increase in both indicates the occurrence of aggregation [50,51]. Samples were prepared as described above for rheological measurement and gelation temperature was also set at 31 °C.

2.6. Surface hydrophobicity measurement

Surface hydrophobicity of skim milk samples was determined fluorometrically (Shimadzu RF-5301PC spectrofluorometer (Shimadzu Corp., Kyoto, Japan)) as described previously [52] using an 8 mM solution of 1-anilinonaphthalene-8-sulfonic acid (ANS) in 0.1 M phosphate buffer (pH 7) as the fluorescent probe at 5 nm for both emission and excitation slit widths and wavelengths of 390 and 470 nm, respectively. Skim milk samples were first diluted with Milli-Q water to stock solutions of 0.01 % protein content and further diluted to 0.0004–0.002 % in 0.1 M phosphate buffer. Measurements were taken 20 min after incubating 4 mL of each dilution with 20 μL of ANS solution in darkness and the intensity of ANS binding to hydrophobic sites was determined based on the slope of the relative fluorescent intensity (RFI) plotted against the protein content. A blank (4 mL buffer + 20 μL ANS) was measured and its RFI taken as the baseline before each set of control (no ANS) or test samples.

Changes in hydrophobic interactions during coagulation were also assessed following a method of Peri et al. [53] with some modifications. Briefly, three portions (20 mL) of each milk sample containing an initial concentration of 0.2 mM ANS were coagulated as described above and the rennet activity in the 1st, 2nd and 3rd portions was stopped after 5, 10 and 20 min, respectively, using 24.4 μL of pepstatin solution (0.99 mM in ethanol). The three renneted portions and 20 mL of the original skim milk (containing 0.2 mM ANS) were centrifuged for 30 min at 10000 rpm and 25 °C and supernatants collected were diluted 40 × with 1 % triton-x100 solution. A calibration curve of RFI (au.) vs. ANS concentration (mM) in 1 % triton-x100 solution was also generated ($y = 162992x + 3.6771$, $R^2 = 0.9998$) and used to estimate the concentration of ANS recovered in each supernatant. Then, the concentration of ANS retained into the curds after 5, 10 and 20 min as a percentage of initially added to milk was calculated and considered a quantitative index of hydrophobic interactions that occurred within each sample after different time intervals.

2.7. Statistics

Results of replicated measurements for each milk or whey sample were submitted to one-way analysis of variance (ANOVA) and comparisons were made by performing the Turkey test using a General Linear Model Procedure of the statistical analysis software (SAS Instrument, 1996). The level of statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Total solids, ash and Ca content in milk and whey and their retention into curds

As shown in Table 1, the concentration of concentrated skim milk samples was >2 times that of the control. Ca content of the control is in line with previously reported [54]. However, serum to total Ca ratio and all Ca fractions' ratios to total protein content appeared to be higher in the control than the concentrated milk. This observation is also consistent with previous studies such as Ferrer, Hill & Corredig [55]. As expected, whey from concentrated skim milk contained higher levels of solids and Ca compared to the control especially during combined renneting and acidification where GDL caused a significantly greater amount of Ca release. This appears in agreement with the previous report by Salvatore, Pirisi & Corredig [20]. As indicated in Table 2, the retention of total Ca and moisture into combined rennet curds (with GDL) were less than in those produced without GDL. During renneting only (without GDL), all constituents tested were apparently retained at a lower rate in control compared to concentrated milk curds.

3.2. Diffusion coefficients, particle size and charge density of milk samples

Diffusion coefficients were 0.9 μ^2/s for concentrated skim milk and 2.1 μ^2/s for control milk samples (Table 3). As diffusion coefficients depend on both viscosity and particle size [56] lower values in concentrated samples may reflect greater viscosity and presence of larger micelles. Indeed, the estimated hydrodynamic diameter (d_h) from these values indicates that concentrated skim milk samples contain larger micelles than the controls. In contrast, it was found that the control milk had greater (>3 ×) absolute value of negative charge density than concentrated skim milk samples.

3.3. Curd formation and structural properties

As expected, concentrated milk in which rennet was added based on protein content had shorter RCT and k_{35} and greater G'_{30} for both sets (with & without GDL) than those of the control (Table 4). Without GDL, the G'_{30} of both control and concentrated milk curds with the same amount of rennet were <1 Pa and both the RCT and k_{35} of the control were >60 min. During combined renneting and acidification (with GDL), however, the RCT of the control was shorter than that of concentrated milk with the same amount of rennet as the control, whereas the difference between k_{35} was not significant ($p > 0.05$). The same trend in coagulation times was also observed from dynamic light scattering measurements based on PDI_1.

Unlike the initial stages of curd formation, the final curd firmness of concentrated milk with the same amount of rennet as the control was higher than that of the control due to a greater curd firming rate as reflected by a steeper increase in G' (Fig. 1A). Tan δ remained stable throughout the primary phase (not shown as it makes the important part (Fig. 1B) unclear) until milk coagulation began where values declined continuously to < 1. Tan δ of the control (with GDL) reached a minimum and rose at a faster rate compared to concentrated milk curds (with GDL) which showed a plateauing behaviour above the control (Fig. 1B). However, tan δ of the control (without GDL) did not reach a minimum value as a thick gel had not formed yet while those of concentrated milk curds (without GDL) were lower than those observed in presence of GDL.

All curds exhibited a solid-like viscoelastic character as G' was greater than G'' while both G' and tan δ were frequency-dependent [57] with a pseudo plastic shear thinning behaviour at low frequency regimes. However, as shown in Fig. 2A–D, the frequency-dependency of the viscoelastic properties was remarkably able to categorise the curds into distinct groups which could be described mechanically as rigid (concentrated milk with rennet added based on protein), hard (concentrated milk with GDL & the same

Table 1
Total solids, ash and Ca fractions in control and concentrated skim milk samples and their respective wheys produced by combined renneting and slow acidification (using GDL) or renneting only.

Sample type	Coagulant	Total solids (mg/g)	Ash (mg/g)	Total Ca (mg/g)	Serum Ca (mg/g)	Ionic Ca (mg/g)
Control milk	NA	90.5 ± 1.5^{E}	7.1 ± 0.0^{E}	1.16 ± 0.01^{D}	0.39 ± 0.01^{E}	0.08 ± 0.00^{E}
Control whey	rennet only	64.9 ± 0.4^{G}	4.9 ± 0.2^{G}	0.35 ± 0.01^{G}	0.35 ± 0.00^{F}	0.06 ± 0.00^{F}
	rennet & GDL	71.8 ± 1.1^{F}	5.7 ± 0.1^{F}	0.64 ± 0.01^{F}	0.63 ± 0.01^{D}	0.25 ± 0.01^{C}
Concentrated milk	NA	246.8 ± 10.3^{A}	18.5 ± 0.1^{A}	2.81 ± 0.04^{A}	0.69 ± 0.01^{C}	0.13 ± 0.00^{D}
Concentrated milk Whey	same rennet as control & GDL	204.7 ± 8.0^{B}	15.2 ± 0.0^{B}	1.65 ± 0.02^{C}	1.62 ± 0.02^{B}	0.34 ± 0.00^{B}
	rennet & GDL based on protein content	204.3 ± 24.2^{B}	14.6 ± 1.3^{C}	1.71 ± 0.01^{B}	1.69 ± 0.02^{A}	0.36 ± 0.00^{A}
	same rennet as control	197.3 ± 11.6^{C}	12.5 ± 0.0^{D}	0.72 ± 0.00^{E}	0.71 ± 0.01^{C}	0.06 ± 0.00^{F}
	rennet based on protein content	190.6 ± 7.7^{D}	12.5 ± 0.4^{D}	0.70 ± 0.01^{E}	0.70 ± 0.01^{C}	0.06 ± 0.00^{F}

Results are presented as means of duplicate (total solids & ash) and triplicate (Ca fractions) measurements ($n \geq 2$) ± standard deviation.
Means in the same column with different superscripts are significantly different ($p < 0.05$).
NA: Not applicable.

Table 2
Retention (% of the original amount in milk) of total solids, moisture, ash and different Ca fractions into curds produced from control and concentrated skim milk samples by combined renneting and slow acidification (using GDL) or renneting only.

Milk type	Coagulant	Total solids (%)	Moisture (%)	Ash (%)	Total Ca (%)
Control	rennet & GDL	33.4 ± 0.5^{B}	6.1 ± 1.5^{C}	28.3 ± 1.6^{E}	50.3 ± 0.1^{F}
	rennet only	36.0 ± 1.7^{B}	8.4 ± 0.2^{C}	38.3 ± 0.3^{C}	75.3 ± 0.2^{C}
Concentrated	same rennet as control & GDL	36.1 ± 1.5^{B}	20.4 ± 2.4^{B}	33.8 ± 1.1^{D}	54.7 ± 0.2^{D}
	rennet & GDL based on protein content	40.4 ± 5.8^{B}	22.4 ± 4.9^{B}	37.9 ± 4.3^{C}	53.3 ± 0.3^{E}
	same rennet as control	54.3 ± 2.4^{A}	43.3 ± 0.8^{A}	54.9 ± 8.4^{AB}	86.0 ± 0.1^{B}
	rennet based on protein content	58.8 ± 4.1^{A}	48.1 ± 1.3^{A}	66.6 ± 5.0^{A}	86.7 ± 0.0^{A}

Results are presented as means of duplicate (total solids, moisture & ash) and triplicate (Ca) measurements ($n \geq 2$) ± standard deviation.
Means in the same column with different superscripts are significantly different ($p < 0.05$).

Table 3
Diffusion coefficients (D), estimated hydrodynamic diameter (d_h) and charge density of reconstituted skim milk samples.

Milk type	D (μ^2/s)	d_h (μm)	Charge density (mV/μm)
Control	2.1 ± 0.02^{A}	0.23 ± 0.00^{B}	-57.2 ± 1.4^{B}
Concentrated	0.9 ± 0.02^{B}	0.34 ± 0.01^{A}	-18.3 ± 0.6^{A}

Results are presented as means of at least duplicate measurements ($n \geq 2$) ± standard deviation.
Means in the same column with different superscripts are significantly different ($p < 0.05$).

Table 4
Gelation properties of control and concentrated skim milk samples under combined renneting and slow acidification (using GDL) or renneting only.

Milk type	Coagulant	RCT (min)	k_{35} (min)	G'_{30} (Pa)	PDI_1 (min)
Control	rennet & GDL	9.0 ± 0.5^{E}	18.8 ± 2.1^{C}	81.0 ± 15.1^{C}	9.4 ± 0.0^{E}
	rennet only	$>60^{A}$	$>60^{A}$	$<1^{D}$	45.9 ± 4.5^{A}
Concentrated	same rennet as control & GDL	14.4 ± 0.6^{C}	17.6 ± 0.6^{C}	333.5 ± 47.4^{B}	14.7 ± 0.4^{C}
	rennet & GDL based on protein content	5.9 ± 0.7^{F}	8.1 ± 1.3^{D}	583.5 ± 68.1^{A}	6.7 ± 0.4^{F}
	same rennet as control	35.4 ± 2.2^{B}	44.5 ± 2.4^{B}	$<1^{D}$	32.7 ± 2.5^{B}
	rennet based on protein content	11.2 ± 0.3^{D}	15.3 ± 0.5^{C}	226.5 ± 27.6^{B}	10.5 ± 0.4^{DE}

Results are presented as means of duplicate measurements ($n = 2$) ± standard deviation.
Means in the same column with different superscripts are significantly different ($p < 0.05$).

rennet as control), soft (control with GDL & concentrated milk with the same rennet as control without GDL) and very soft (control without GDL). The latter was completely deformed and returned to liquid milk-like state at frequencies ≥6.8 Hz. An increase in G', η^* and τ at higher frequencies indicates a network recovery probably due to limited time for bonds to relax [58] and new cluster formation by hydrodynamic interactions. The concentrated milk curd with GDL & the same rennet as control was not categorised as rigid because at >68 Hz tan δ increased to ~1/3 of the original value (i.e., $G'' > G'$). Soft curds were also significantly affected as shown by the rise in tan δ and η^*, but were not deformed completely until the frequency reached 100 Hz. The τ values of all samples increased with frequency, showing that more stress was required to maintain a constant strain.

Measurement of changes in diffusion coefficients during curd formation complemented rheological measurements. A synergistic action of rennet and GDL on promoting a rapid coagulation in combined gels was evident (Fig. 3A) compared with slow coagulation by renneting only (Fig. 3B). The three phases of curd formation could also be clearly shown: (1) slight increase in diffusion due to simultaneous enzymatic hydrolysis of κ-casein and dissolution of micellar $Ca_3(PO_4)_2$ caused by acidification, the opposite trend with the same interpretation was also seen previously for changes in particle size [9,38,42,51], (2) sharp drop due to aggregation of para-casein up to a minimum at PDI_1 (flocculation) indicating a condensed phase or sol-gel transition state and (3) slight rise which was much more apparent in combined control curd, an indication of extensive restructuring, and whose rate decreased as the curd strengthens and ages. An almost linear increase in diffusion coefficients ($R^2 = 0.74$) in the third phase of the control curd during combined renneting and acidification was observed. A similar change both with time and decreasing pH was also observed during acidification without rennet (Fig. 4A_1 & B_1). On the other hand, concentrated milk exhibited a slight change despite similar degree of acidification to that of control (Fig. 4A_2 & B_2).

3.4. Changes in hydrophobic interactions

A linear plot of RFI vs. protein content (figure not shown) indicates that the number of hydrophobic sites increased parallel to the milk concentration. The percentage of initial concentration of the fluorescent probe (ANS) retained into concentrated milk curds during coagulation was also found to be greater than observed in control curds (Fig. 5). The ANS retention appeared at ≥70 % for concentrated milk and 40 % for control with GDL, whereas control without GDL showed only 8 % retention of the initial ANS 20 min after addition of coagulants. It is also clear that the curves of ANS retention rose sharply immediately after adding coagulants, reaching

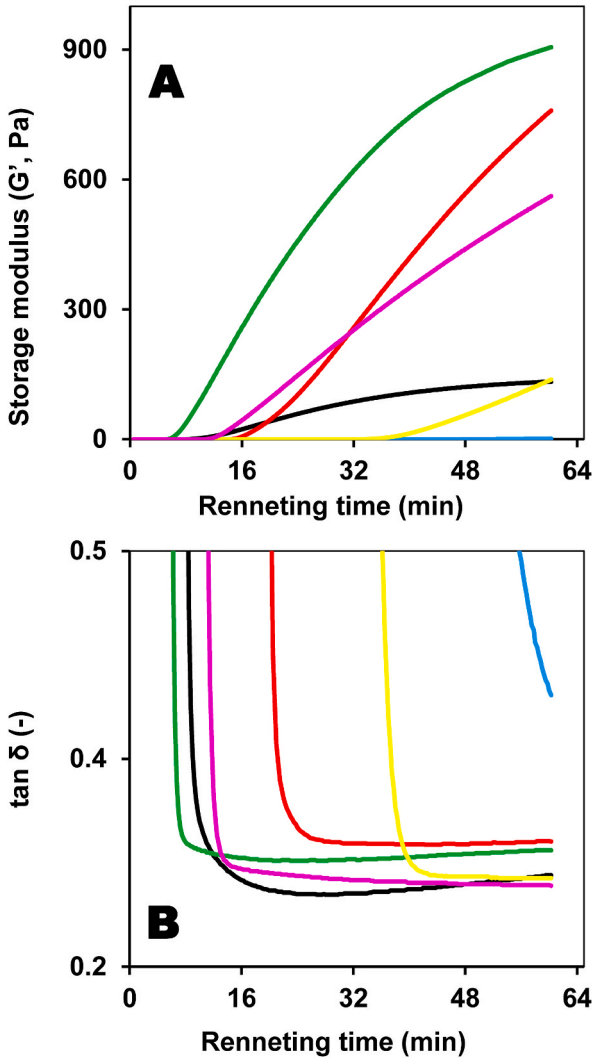

Fig. 1. Changes in G' (A) and tan δ (B) with time during rennet coagulation of skim milk samples (black line: control with GDL; blue line: control without GDL; green line: concentrated milk with GDL & rennet added based on protein content; purple line: concentrated milk without GDL & rennet added based on protein content; red line: concentrated milk with GDL & same rennet as control; yellow line: concentrated milk without GDL & same rennet as control).

a plateau within 5 min for samples containing GDL, and 10 min for concentrated milk without GDL while that of control without GDL showed a small increase only for a 20 min sub-sample. Similar results were reported [53]. This clearly explains that a slower curd firming rate observed from rheological and light scattering measurements was partly due to limited hydrophobic interactions and demonstrates their key role during early stages of curd formation. Moreover, the influence of milk concentration was much higher compared to that of pH.

4. Discussion

Concentrated milk systems are widely applied in cheese industries for cheese milk standardisation which helps to achieve consistent cheese composition and texture, and have the potential to improve cheese yield. Here, a series of changes occurring within the gel matrix under two rennet and acidification levels were assessed. Concentrated skim milk renneted based on protein content clotted faster due to an increased micellar size indicated by higher d_h (Table 3) usually as a consequence of greater sticking probability [59], and sufficient rennet/casein ratio. Although a certain proportion of κ-casein needs to be hydrolysed before clotting begins [60, 61], this stage appeared to have no significant effect on RCT compared to that of acidification. However, G'_{30} of concentrated milk (Table 4) indicates the dependence of flocculation time and curd firming rate on the renneting extent and milk concentration, respectively, which is in strong agreement with Karlsson, Ipsen & Ardö [62].

In absence of an acidifying agent, low charge density, greater amount of soluble Ca fractions (Table 1) and increased hydrophobic

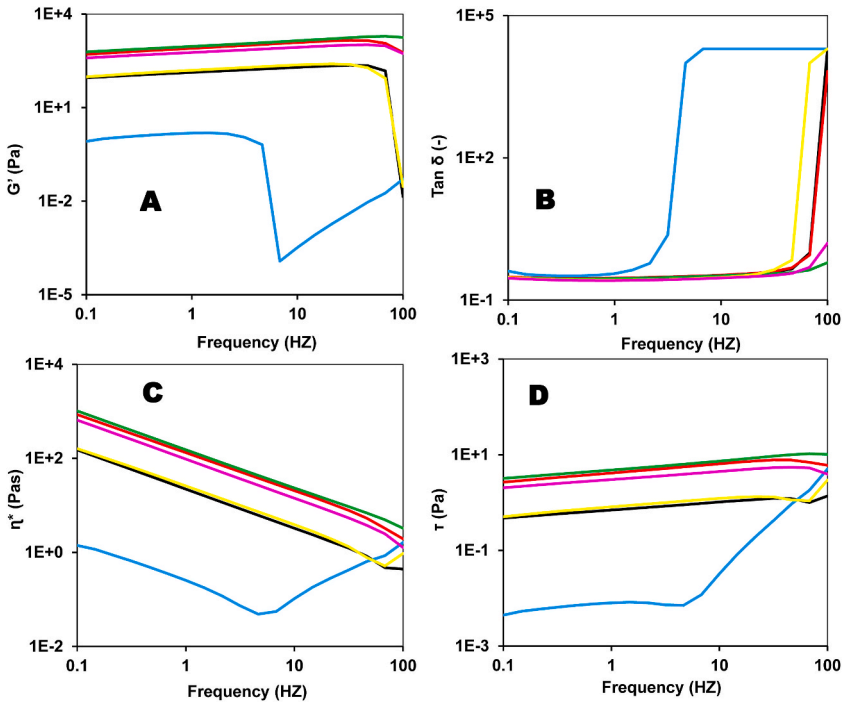

Fig. 2. (A) Storage modulus (G'), (B) tan δ, (C) complex viscosity (η*) and (D) shear stress (τ) as a function of oscillatory frequency for rennet coagulated skim milk curds (black line: control with GDL; blue line: control without GDL; green line: concentrated milk with GDL & rennet added based on protein content; purple line: concentrated milk without GDL & rennet added based on protein content; red line: concentrated milk with GDL & same rennet as control; yellow line: concentrated milk without GDL & same rennet as control).

interactions (Fig. 5) appear to have played a significant role in addition to a greater micellar size aforementioned. The rate of change in hydrophobic interactions observed in combined gels (within 5 min) demonstrates the role of pH reduction (from 6.8 for control or 6.4 for concentrated samples to 6.0 ± 0.1) in minimising the clotting time and increasing the curd firming rate and curd strength (Fig. 1A). This probably relies on a strong synergistic action of both coagulants towards reducing the charge and chain densities [42] which effectively drops the net repulsive forces or energy barrier between micelles, while stronger gels (higher G′) could be attributed to a highly branched and interconnected network [63].

According to Mellema [12], higher G′ values suggest a great number and strength of junctions between particles which increases the local compactness and size of the compact building blocks. It was also found that tan δ, spontaneous syneresis and pore sizes increase with decreasing pH [5]. However, despite higher G′ in combined gels from concentrated milk (Fig. 2A), higher tan δ (Fig. 2B), which accounts for conditions that promote structural changes, shows that concentrated milk curds have relatively weakly organised structures. Lucey et al. [6] suggested that high or low tan δ values are indicators of extensive or fewer large scale structural rearrangements, respectively. It had also been proposed [59] that a high tan δ value is an indication of shorter bonds relaxation time due to flow-like rearrangements. This would imply that concentrated milk curds underwent greater rearrangements than the control. In contrast, Karlsson et al. [62] reported a lower extent of rearrangements in highly concentrated UF skim milk curd, and this could be related to the concentration method. In the present study, an apparently continuous rise in tan δ in the later phase of the control compared to a plateauing behaviour shown by concentrated milk curds, is also a likely indication of more bond relaxation and a tendency for syneresis as suggested by van Vliet et al. [64], i.e., progressive rise in rearrangements and microphase separation due to changes in type and strength of bonds being formed during curd ageing.

Progressively higher structural rearrangements during curd ageing in combined control were confirmed by a continuous rise in diffusion coefficients (Fig. 3A). In acidified gels, demineralisation of colloidal $Ca_3(PO_4)_2$ [65] reduces the resistance to deformation due to lower bond energy and promotes restructuring and microsyneresis. This reflects an increased number of deformable bonds as particles have greater mobility. An important structural rearrangement taking place during curd ageing is particle fusion [12]. This leads to a progressive coarsening of gel structure and pore size increments that promote phase separation. Unlike controls, however, highly concentrated curds exhibit limited macroscopic contraction [66], thus, limited cluster fusion and macrosyneresis. The overall curd is stiff due to a higher solid to moisture ratio [67], but the rate of whey separation during curd ageing is likely greater in control. Therefore, as changes in diffusion coefficients in concentrated milk curds were similar regardless of pH (Fig. 3), they likely rather undergo slow microsyneresis and long-term rearrangements at bonding sites (primary particle level) due to an increasing number of unbonded particles, and perhaps responsible for more flow-like behaviour shown in Fig. 1B.

It is thought that the gel strength is strongly reduced by increasing the size of the primary aggregates [68], besides, milk from cows known to naturally have small casein micelles produce firmer gels [9,69]. According to Mezzenga & Fischer [70], the size of interacting

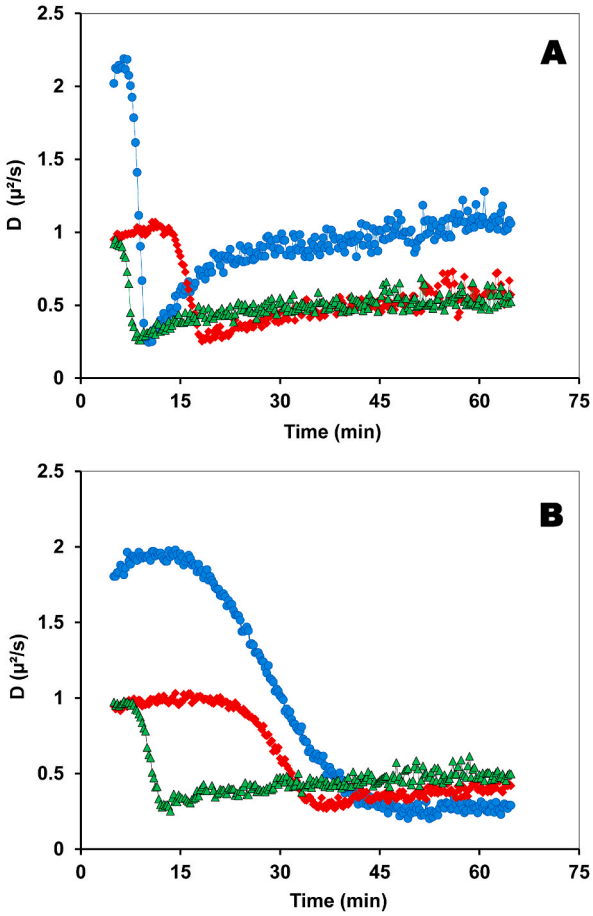

Fig. 3. Changes in diffusion coefficients (D) as a function of gelation time during renneting combined with acidification using GDL (A) or renneting only (B) of control (blue line) and concentrated skim milk samples with the same rennet as control (red line) or with rennet added based on protein content (green line).

colloids causes a large competition between electrostatic repulsive and hydrophobic attraction forces. However, small native micelles are usually associated with higher levels of κ-casein [71]. Moreover, it is clearly shown herein that the gel strength increased with milk concentration despite greater hydrodynamic radius of casein micelles in milk. Therefore, an estimated increase in hydrodynamic radius due to milk concentration appears one of the essential changes resulting from micellar restructuring. Micellar restructuring arises from shorter inter-particle distance and altered ionic equilibrium creating an imbalance in net repulsive forces causing some of the serum Ca and caseins to shift into the colloidal phase and some of micelles to partially adjoin [26,30] as repulsive forces are overcome by attraction. This is supported by low diffusion coefficients obtained (Table 3) which are not only indicative of greater hydrodynamic radius but also increased attractive protein-protein interactions [72]. Eventually the charge density also decreases (Table 3), contributing to the rise in surface hydrophobicity which plays a key role. Therefore, it may be this micellar restructuring-related changes that determine the type and strength of interactions formed during gelation and rearrangements, which according to Dickinson [73] the gel structure and pore size depend on. He argued that the pore size is particularly determined by the degree of attractive interconnections and phase separation.

In the current research, the rate of attractive rearrangements (specifically, floc contraction) and macroscopic phase separation during curd ageing in concentrated milk gels appears to be limited. This explains a higher moisture retention (Table 2) as an indication of impaired macrosyneresis. Higher retention of Ca and other solids may also be related to more serum solids associated with water retention rather than to greater Ca binding. In contrast, a significant decrease in curd moisture on increasing milk concentration up to 4 % [74], 4.6 % [16] or 6 % protein [19] was reported, showing that curd structure improves under optimum limits of milk concentration and explained by microstructural changes such as pore size [75]. However, fractures were observed in process cheese from concentrated milk up to 6 % protein which resulted in the failure to entrap fat [76], an indication of a less-cohesive matrix [11].

5. Conclusion

This study examined how skim milk total solids concentration modulates the rennet curd structure using dynamic rheometry,

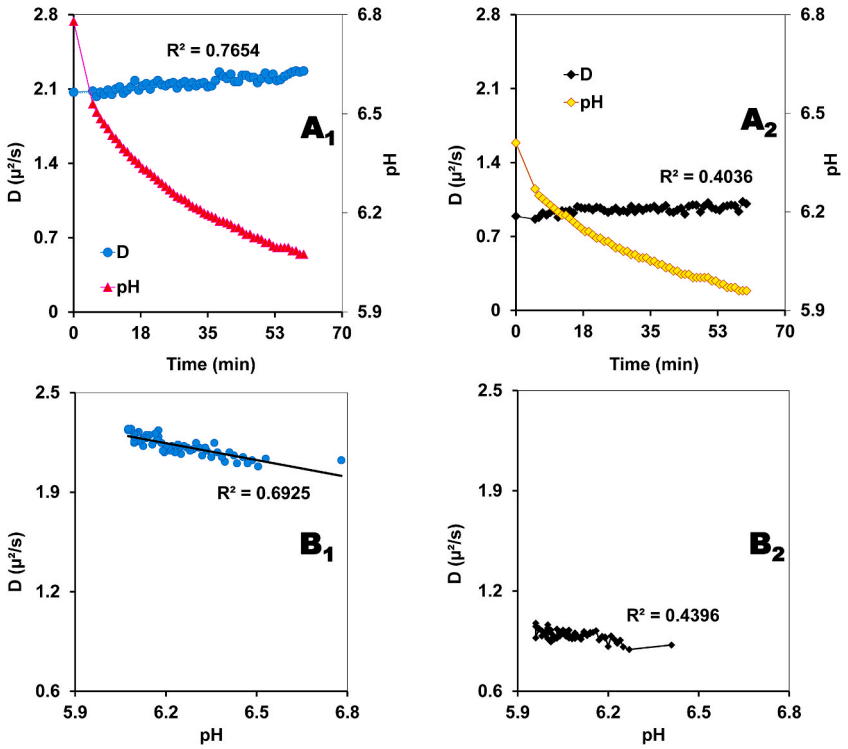

Fig. 4. Changes in diffusion coefficients (D) and pH as a function of time during 1 h of acidification using GDL for control (A_1) and concentrated (A_2) skim milk samples as well as changes in D with pH during 1 h of acidification using GDL for control (B_1) and concentrated (B_2) skim milk samples.

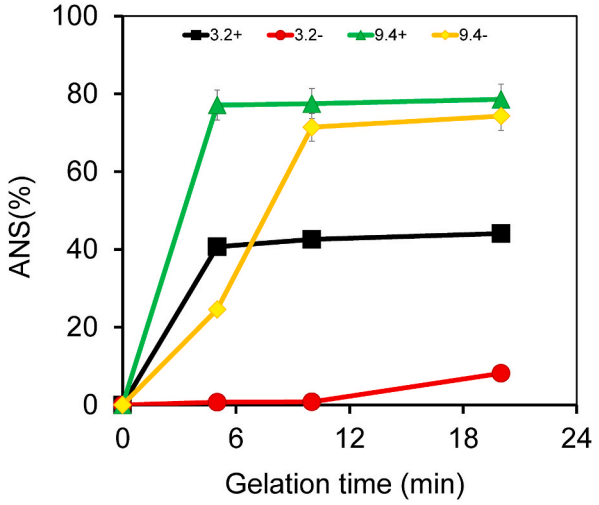

Fig. 5. ANS retained into the curds (% of initial concentration added to milk) at different time intervals during rennet coagulation of the control skim milk with GDL (black line with squares) or without GDL (red line with circles) and concentrated skim milk samples with GDL (green line with triangles) or without GDL (yellow line with rotated squares).

dynamic light scattering and fluorimetry. It is concluded that micellar restructuring induced by the change of net repulsion – as evidenced by increased micellar size, lower serum/total Ca ratio, charge density and diffusivity, and greater hydrophobicity – is the underlying reason for variations in rennet curd formation and ageing properties due to milk concentration. This paves the way for future research into the drivers of curd formation and matrix integrity, enzyme kinetics, and caseins, fat and rennet activity partitioning, which may help to better control yield, texture and maturation rate of cheeses made from milks of different concentrations or protein genetic variants. The key driver could probably be the change in the predominant type and/or strength of major casein

interactions which can be established by performing specific interactions/bond blocking or dissociation tests.

Funding statement

This study did not receive any specific funding.

Data availability statement

The data used are contained in tables and figures within the article.

CRediT authorship contribution statement

Joseph F. Kayihura: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The author declares no competing interests.

Acknowledgements

The support of Commonwealth Government of Australia through Australian Government Research Training Program Scholarship is highly appreciated.

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